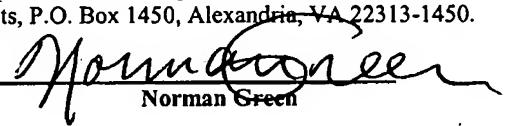


IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Patent Application of:	Group Art Unit: 1643
Applicants: Sierra <i>et al.</i>	Confirmation No.: 4354
Serial No.: 10/003,462	Examiner: Anne Holleran
Filed: December 6, 2001	<u>Certificate of Electronic Filing</u>
Title: VACCINE COMPOSITION CONTAINING TRANSFORMING GROWTH FACTOR ALPHA (TGF α). ITS USE IN MALIGNANT DISEASES THERAPY	I hereby certify that the attached Response to the Office Action dated August 8, 2007 and all marked attachments are being deposited by Electronic Filing on February 8, 2008 by using the EFS - Web patent filing system and addressed to: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.
Docket No.: 30797-717.201	By:  Norman Green

Declaration Under 37 CFR § 1.131 of Belinda Sánchez Ramírez

Mail Stop RCE
Commissioner for Patents
PO Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

I, Belinda Sánchez Ramírez, of Havana, Cuba, hereby declare as follows:

1. I am the Head of Recombinant Vaccines Group and a researcher at the Centro de Inmunología Molecular and an inventor of the present application. I have been conducting research in fusion proteins and tumor immunology for over 12 years. Accordingly, my *Curriculum Vitae* is attached herewith as **Exhibit A**.
2. I have read the specification of the above-identified application, the pending claims and the Office Action mailed by the USPTO on August 8, 2007.

3. I understand that the Examiner has rejected the claims as allegedly being obvious in view of several references, one of which is Gonzalez (Gonzalez et al. Scandinavian J. Immunol., 52: 113, August 2000).

4. This reference was published less than a year before the filing date of priority application CUBA 286/2000, filed December 6, 2000.

5. The compositions as disclosed and claimed in the present application were conceived and reduced to practice prior to August 2000.

6. As evidence of this, attached herewith as **Exhibit B** is a laboratory notebook page exemplifying the protocol used to generate P64-TGF α fusion protein as described in Examples 2 and 3 of the present application.

Briefly, the expression vector pM 92 was used. The plasmid contains the *IpdA* gene coding for P64k protein from *Neisseria meningitidis* (strain B385) under the control of *E. coli* tryptophan operon promoter (*ptrp*) and phage T4 transcriptional terminator (*tT4*). pM92 contains ampicillin (Amp^R) and kanamycin (Km^R) antibiotic resistance expression cassettes. The pM92 vector was digested and subsequently ligated with the cDNA from TGF α .

The resulting plasmid codes for the fusion protein that contains hTGF α inserted among the amino acid 45/46 of P64k and containing a polyHis sequence.

FIG. 2 of the present application shows a schematic representation of the expression vector obtaining process. This vector codes for the fusion protein TGF α -P64K which was made using techniques described in the laboratory notebook page and herein.

7. In summary, the laboratory notebook page presented herein illustrates that the TGF α -P64K fusion protein compositions as presently claimed were conceived and reduced to practice prior to August 2000.

8. Also attached herewith is **Exhibit C**, which is a copy of Silva et al., FEMS Microbiology Letters, 174: 191-199 (1999) showing that the IpdA gene encodes P64K (see abstract & introduction, pp. 1919-92).

9. **Exhibit D**, also filed herewith, is a copy of two additional pages from the inventors' laboratory notebook. These notebook pages show that the plasmid pM92 and pMTGF contain the IpdA gene. The first page (page 14, dated May 13, 1999 in the upper right-hand corner) describes the pMTGF plasmid and the expression of the TGF α -P64K fusion protein encoded by this plasmid. The plasmid map for pM92 is found on the next page of the Exhibit. The plasmid map clearly identifies the plasmid pM92 (see upper right plasmid) and shows that it contains the IpdA gene. The plasmid pSK contains the gene for human TGF α (see upper left plasmid), and the pMTGF plasmid contains the fusion protein between TGF α and the IpdA gene. In the final plasmid, pMTGF, the TGF α gene is clearly identified as fused with the IpdA gene.

10. In summary, the IpdA gene is known to encode the P64K protein of *Neisseria meningitidis* and the laboratory notebook pages conclusively demonstrate the conception and reduction to practice of the TGF α -P64K fusion protein compositions as presently prior to the August 2000 publication date of Gonzalez et al.

11. Also attached herewith as **Exhibit E** is a second laboratory notebook page demonstrating conception and possession of the TGF α -P64K fusion protein by the inventors prior to the August 2000 publication of Gonzalez et al. In particular, page 23 of the laboratory notebook (dated August 13, 1999 in the upper right-hand corner of the page) describes the expression of the TGF α -P64K fusion protein from pMTGF (described above and in Exhibit B) in *E. coli* bacterial cells. The description of the figure at the bottom of the page explicitly identifies the expression of the fusion protein "TGFalpha-P64K" in *E. coli*, referring to the expression of TGF α -P64K fusion protein encoded by the pMTGF plasmid.

12. In summary, this laboratory notebook page provides additional evidence of the conception and reduction to practice of the TGF α -P64K fusion protein compositions as presently claimed prior to the August 2000 publication date of Gonzalez et al.

13. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section § 1001 of Title XVIII of the United States Code and that willful false statements may jeopardize the validity of this Application for Patent or any patent issuing thereon.

Belinda Sánchez Ramírez

Date: 22.04.2008

Signature: B.S.R.

Exhibit A

Curriculum Vitae

Personal data

Name: Belinda Sánchez Ramírez
Date of birth: January 30, 1970
Citizenship: Cuban
Home address: 184 Street between 1st and 5th Ave, Flores, Playa
Havana 11600, Cuba

Present position: Researcher of Vaccine Department.
Head of Recombinant Vaccines Group
Research and Development Direction.

Present Address: Center of Molecular Immunology (CIM), 216 Street and 15,
Atabey, Playa. Havana 11600, P.O. Box 16040, Cuba.
Telephone: 537 217645 Fax: 537 335049
E.mail: belinda@ict.cim.sld.cu

Education: BS in Biochemistry with Distinction (Faculty of Biology, Havana
University, 1987-1992).

Professional Experience: Center of Molecular Immunology (1992 – present).

- Technical experience in biochemical techniques (SDS-PAGE, Western Blott), Molecular Biology, cell culture, protein purification.
- Generation of a cancer vaccine based on human EGF, chemically conjugated to P64k from *Neisseria Meningitidis* and evaluation in preclinical studies (CIM).
- Cloning, expression and purification of a fusion protein human EGF-P64k from *Neisseria Meningitidis* for the formulation of cancer vaccine. Evaluation of the immunogenicity in preclinical studies (CIM).
- Cloning, expression in mammalian cells and purification of extracellular domain of murine EGFR (mEGFR-ECD) and human EGF receptor (HER1-ECD). (Max-Plank Institute for Biochemistry, Germany).
- Generation of a cancer vaccine based on mEGFR-ECD and Her1-ECD adjuvated on very small size proteoliposomes from *Neisseria Meningitidis*. Evaluation of the humoral and cellular immunogenicity and antitumoral effect in preclinical studies (CIM)

Post-Graduate Studies

- Molecular Immunology course (CIM / University of Havana, 1992).
- Applied Biotechnology Course (CIM / University of Havana, 1993).
- Immunology Course (CIGB, 1993).
- Quality Control and Statistic (CIM / ISPJAE, 1994)
- Good manufacturing Practices (CIM, 1994).
- Immunology Course (CIM, 1995).
- English Course (University of Havana, 1996).
- German Course (Goethe Institute, Freiburg, Germany, 1996).
- Molecular Oncology Course (Havana, Cuba, 2000)
- Introduction to proteomic (Havana, Cuba, 2006)

Participation in meetings and Congress:

- Biotechnology Congress Havana'92. CIGB, Havana, Cuba, 1992.
- First International Symposium about Encephalic Death. Nefrology Institute, Havana, Cuba, 1992.
- Second national Workshop of Cellular Immunity. Finlay Institute, Havana, Cuba, 1994. Author.
- International Workshop "Immunotherapy in the Nineties". CIM, Havana, Cuba, 1994.
- XII Scientific Seminar "Cancer Immunology and Immunotherapy ". CNIC, Havana, Cuba, 1995. Author.
- International Workshop "Immunotherapy in the Nineties". CIM, Havana, Cuba, 1996. Co-author.
- International Workshop Biotechnology 1997 "Medical applications of biotechnology" (CIGB, Cuba, 1997)
- XI Forum of Science and Technology, Havana, Cuba, 1997
- Forum of Science and Technique. CIM, Havana, Cuba, 1997 and 1998. Co-author.
- "Cancer Vaccines'98" Conference. Bethesda MD, USA. Co-author.
- XIII Latin-American Integrated Congress of Cancerology. Havana, Cuba, 1999. Author and co-author.
- Workshop "Immunotherapy for the New Century, Havana, Cuba, 2000.
- 6th Latin-American Congress of Immunology. Havana, Cuba, 2002
- International Workshop: Immunotherapy for the New Century, Cuba, 2002.
- Biotechnology Congress Havana '2003. Havana, Cuba, 2003.
- International Workshop: Immunotherapy for the New Century, Cuba, 2004.
- Cancer Vaccines/Adjuvants/Delivery for the Next Decade Congress(CVADD), Portugal, 2005.
- Cuban National Immunology Congress, Cuba, 2006.
- International Workshop: Immunotherapy for the New Century, Cuba, 2006
- Workshop UCL-CIM, 2007

Docent experience

- Professor of Advanced Molecular Immunology Course in 2002, 2004, 2006 and 2007.
- Supervisor of diploma thesis in 2001 and 2006.

Publications and patents

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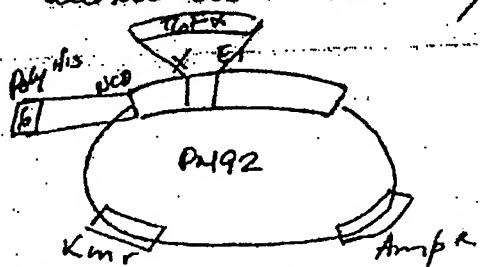
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Exhibit B

Jueves:

③ CHEQUEO HIND III Clonaje pM92 - T6FK (vector flaco).
 La cosa quedó ox pues el pM92 debe sacar bandas 3,3 y 2,89 y
 los clones 2. too 3,3 y 3,00 y en los minis se ve una sola
 banda (migran juntas) y en el pM92 se ven 2 bandas.

ojo: El asunto del clonaje del T6F-X se complicó por lo siguiente:
 El objetivo es clonar el T6F-X en Xba I/ECOR I del pM92
 y después clonarse una cola de poly his en el sitio NCO I
 delante del N-tetracinaff P64K.



y hasta ahora se tiene clonada la
 banda del T6FK X/E1 en el pM92
 pero ~~en el pM92~~
 → no se puede clonar
 la cola de poly his x digestión
 simple

Primer clonaje

Clonarle cola de poly-his

↓
 pM92 (his) / Xba I / ECOR I

↓

Clonarle el T6FK

1- Primer clonaje

pM92 + cola his

Digestión pM92 NCO I

22 ul pM92 6C (v 3 ug)

10 ul b4NEB

30 ul NCO I (10 u (ul))

58 ul H₂O

100 uls

Exhibit C

Characterisation of the *lpdA* gene from *Neisseria meningitidis* by polymerase chain reaction, restriction fragment length polymorphism and sequencing

Ricardo Silva *, Tamara Menéndez, Luis M. Alonso, Enrique Iglesias, Alexis Musacchio, María de Jesús Leal, Anabel Alvarez, Edelgis Coizeau, Alejandro Martín, Luis Herrera, Gerardo Guillén

División de Vacunas, Centro de Ingeniería Genética y Biotecnología, Ave. 31, entre 158 y 190, Apartado 6162, CP 10600, La Habana, Cuba

Received 25 December 1998; accepted 8 January 1999

Abstract

P64k protein from *Neisseria meningitidis* is well recognised in sera from individuals convalescent from meningococcal disease or vaccinated with the Cuban antimeningococcal vaccine VA-MENGOC-BC®. The presence of the protein in more than 80 meningococcal strains has also been verified. It is immunogenic in animal models and the antibodies elicited show bactericidal activity against meningococci. To further investigate at the molecular level whether *lpdA*, the gene coding for P64k protein, is conserved among different *N. meningitidis* strains, a total of 20 strains isolated from different geographic areas were differentiated on the basis of restriction fragment length polymorphism (RFLP) patterns after polymerase chain reaction (PCR) amplification of the *lpdA* gene and restriction endonuclease digestion with *Hpa*II. Although a total of five different PCR-RFLP patterns were present, nucleotide sequence determination showed that identity levels were as high as 93–99% among the *N. meningitidis* strains analysed. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: *Neisseria meningitidis*; *lpdA* gene; Polymerase chain reaction; Restriction fragment length polymorphism

1. Introduction

Neisseria meningitidis is the causative agent of meningococcal meningitis. This is an important health problem throughout the world. Based on the antigenic differences in their capsular polysaccharides (CPS) 13 serogroups of *N. meningitidis* have been

identified. Serogroup A is responsible for large outbreaks world-wide, serogroup B is responsible for most cases of meningococcal meningitis in Europe, South Australia and America, while serogroup C occurs sporadically and is seen in local outbreaks [1–3]. The currently available vaccines against serogroups A, C, W-135, and Y consist of the purified CPS of these serogroups. These vaccines fail to invoke an adequate immune response in young children and do not elicit memory response [4,5].

In the case of serogroup B, vaccination ap-

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proaches have included the use of outer membrane vesicles (OMV) in which outer membrane proteins (OMPs) are major components [6–8]. One of such OMV-based vaccines is the Cuban VA-MENGOC-BC® vaccine [6]. Its introduction into the Cuban National Immunisation Program has decreased the incidence of meningococcal disease in Cuba between 1983 and 1994, from 14.4 to 0.45 cases per 100 000 persons [9].

There are five main classes (classes 1–5) of OMPs in *N. meningitidis* [10]. With the exception of class 4, the remaining classes are considered attractive candidate vaccine antigens [1–3]. In addition, class 2 and 3 OMPs define the serotypes [11,12], while class 1 proteins are targets of the serosubtyping antibodies [13].

Besides the major OMPs, the characterisation of other proteins of *N. meningitidis* may constitute an attractive strategy for vaccine development against meningococcal disease. Our group has previously identified a meningococcal 64-kDa molecular mass protein named P64k. Its coding gene, *lpdA*, was isolated, cloned and expressed in *Escherichia coli*. The gene and the protein have been sequenced and the three dimensional structure of P64k has been determined. P64k seems to be the same protein as the dihydrolipoyl dehydrogenase (E₃) protein, component of pyruvate dehydrogenase complex, characterised by Ala'Aldeen et al. [14]. Even when the nucleotide and amino acid (aa) sequences display high degree of homology with the dihydrolipoamide dehydrogenases, we postulated that this protein can be considered a potential vaccine candidate for the following reasons: (i) P64k is well recognised in immunoassays by sera from humans convalescent from meningococcal disease or vaccinated with the Cuban antimeningococcal vaccine VA-MENGOC-BC®. (ii) The protein is highly immunogenic in animals and the elicited sera show, although moderate, bactericidal activity against meningococci. (iii) It is expressed by the majority of the meningococcal strains so far examined [15–19]. The questions regarding the pathogenic role of P64k are now being examined by our research group.

To continue the characterisation of this meningococcal antigen we studied the conservation at the molecular level of the *lpdA* gene present in 20 strains of *N. meningitidis* belonging to serologically different

groups, types and subtypes. In the present work the *lpdA* gene is characterised both by RFLP analysis of the PCR-amplified gene, and by DNA sequencing. A comparative analysis of the nucleotide and deduced aa sequences obtained is also presented.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The meningococcal strains used in this study and their serological classification are listed in Table 1. They were grown in brain heart infusion (BHI) agar plates, overnight at 37°C, in a candle jar. For liquid culture, BHI was used. The working stock of each strain was prepared in 10% skimmed milk, and stored at -70°C until required.

E. coli XL1-Blue [*recA1 endA1 gyrA96 thi hsdR17* (*r_k*⁻ *m_k*⁺) *supE44 relA1 λ⁻ lac* (F' *proAB lacI^q* *ZΔM15* *Tn10(tet)*)] [22] competent cells were used for the transformation of recombinants. They were cultured in Luria-Bertani (LB) medium [23], supplemented with ampicillin (50 µg ml⁻¹), when required, overnight at 37°C.

2.2. DNA procedures

Routine recombinant DNA technology was performed as described [23]. Plasmid small-scale purification was carried out by the alkaline extraction method [24]. Chromosomal DNA was extracted as described in Guillén et al. [25].

A Southern blot of *EcoRI/HindIII*-digested chromosomal DNA fragments was performed basically as previously described [26]. As a probe, the 1.8-kb *lpdA* gene from *N. meningitidis* strain B385 was used. This was obtained by PCR amplification under the following conditions: 30 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 2 min. The reaction components were: 1 µg of chromosomal DNA from meningococcal strain B385; 50 pmol of primers 1 (5'-TTGAATT~~C~~ATGGCTT~~T~~AGTTGAATTGAA-AG-3') and 2 (5'-TT~~G~~GGATC~~C~~TTATT~~T~~TTTC TT-TTGC~~G~~GGAGG-3'); 200 µM of each deoxynucleotide triphosphates (dNTPs); PCR buffer (10 mM KCl, 20 mM Tris-HCl pH 8.8, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% (v.v) Triton X-100); double-

distilled water to a final volume of 75 µl and 1 unit per reaction of *Thermus aquaticus* DNA polymerase (Heber-Biotec, Havana, Cuba). The primers were designed on the basis of analysis of the nucleotide sequence of the *lpdA* gene from *N. meningitidis* strain B385, as published by Silva et al. [15] and accessible in the EMBL database with the accession number X77920. The probe was labelled using the ECL direct nucleic acid labelling and detection system kit (Amersham, UK). Hybridisation and washing of filters were performed according to the instructions of the manufacturers of this kit. *Saccharomyces cerevisiae* DNA was kindly provided by Dr Verena Muzio from CIGB, Havana, Cuba. The *N. meningitidis* *lpdA* knockout strain [27] carries a completely inactivated copy of *lpdA* in which most of the gene has been replaced by the kanamycin resistance cassette from pUC4K [28], leaving only 100 bp and 200 bp at the 5' and 3' ends, respectively. Expression of *lpdA* in this strain is undetectable by Western blotting using the monoclonal antibody (mAb) 484, specific for the first 5 N-terminal amino acids of the P64k protein, which was kindly provided by Dr Emilio Carpio from the CIGB of Sancti Spiritus, Cuba.

PCR-RFLP was done as follows. The amplification of the *lpdA* gene from the remaining 19 strains of *N. meningitidis* was performed in the same conditions and using the same oligonucleotides described for the strain B385. In each case, 1 µg of chromosomal DNA from each of the analysed meningococcal strains was used. PCR products from the 20 strains were restricted with *Hpa*II and the restriction endonuclease products were loaded onto 10% polyacrylamide gel in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 1 mM EDTA, pH 8.0). The run was done at 200 V for 10 h. If the sum of the lengths of the bands obtained after digestion was approximately equal to the estimated size of the PCR product (~1.8 kb), complete digestion was assumed. DNA was visualised by silver staining.

The primers for the PCR were designed with *Eco*RI (primer 1) and *Bam*HI (primer 2) recognition sites (underlined above) for the cloning of the *Eco*RI/*Bam*HI restricted PCR products into the pUC18 vector [29], previously digested with the same enzymes.

DNA sequencing reactions were performed following the dideoxy-chain termination method [30], using the Sequenase 2.0 kit (Amersham-USB, USA), ac-

cording to the manufacturer's instructions. The reactions were run as described [23] using a Base Runner Nucleic Acid Sequencer apparatus (IBI, USA). The consensus multiple alignments of nucleotide and deduced aa sequences were done using the CLUSTAL W program [31].

2.3. Western blotting

The OMV from *N. meningitidis* were obtained using the LiCl extraction method [11]. 20 µg of each sample, determined following the method of Lowry et al. [32], were loaded per lane and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described [33]. The proteins were transferred from acrylamide gels to 0.45 µm pore size nitrocellulose membranes. The membranes were blocked with skimmed milk. Immunodetection was carried out as in [34], using mAb 114, which is specific for the peptide DAADIGKTIHP present in the P64k protein of *N. meningitidis*, and that was kindly provided by Dr Consuelo Nazábal from the CIGB (Havana, Cuba). The epitope recognised by this mAb was mapped using Pin Technology [35].

3. Results and discussion

In a previous work [15], a high molecular mass protein of *N. meningitidis*, named P64k ($M_r = 64$ kDa), present in the majority of the meningococcal strains examined, was identified. By the construction of a genomic library in the phage EMBL 3, and its screening with sera from OMV-immunised rabbits, we cloned the P64k coding gene. It showed a high degree of homology with the lipoamide dehydrogenase gene from *E. coli*, so it was named *lpdA* [15–17]. We have produced P64k protein at high levels in *E. coli*. The recombinant protein is well recognised in immunoassays by the sera from individuals convalescent from *N. meningitidis* infections or vaccinated with the Cuban antimeningococcal vaccine VAMENGOC-BC® [6,15]. The purified recombinant polypeptide was highly immunogenic in animal models, and it was able to elicit a moderate bactericidal response against meningococci [15,18].

For all the above reasons, we consider that P64k is a potential antimeningococcal vaccine candidate. Be-

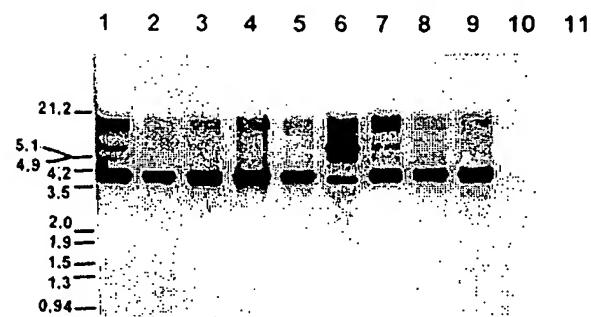


Fig. 1. Analysis on Southern blotting of 2 µg of chromosomal DNA, restricted with *Eco*RI and *Hind*III from nine of the 20 *N. meningitidis* strains analysed in this study. Lane 1: strain B385 (positive control). Lane 2: strain 51/90. Lane 3: strain 102/90. Lane 4: strain 104/90. Lane 5: strain 131/91. Lane 6: strain B16B6. Lane 7: strain M982. Lane 8: strain M990. Lane 9: strain 41/91. Lane 10: *lpdA* mutant strain (negative control). Lane 11: *S. cerevisiae* DNA (negative control). The size markers are indicated on the left in base pairs.

cause of this, our research group is interested in a better characterisation of this protein. Questions relating to its pathogenic role within the bacterium are now undergoing investigation.

In order to design a broad-spectrum vaccine, another important question to be considered is the conservation of the candidate among a great number of meningococcal strains. In this work, we studied the conservation at the molecular level of the *lpdA* gene from 20 meningococcal strains belonging to different serogroups, serotypes and serosubtypes. The strains under analysis and their serological classification are listed in Table 1.

To verify the presence of the *lpdA* gene and its expression in the strains under analysis, Southern and Western blot experiments were carried out. Fig. 1 shows the results of the Southern blot assay in which 2 µg of chromosomal DNA from the 20 meningococcal strains were restricted with *Eco*RI and *Hind*III and hybridised with the *lpdA* gene of

Table 1
Meningococcal strains used in this study, geographical origin and their serological classification, PCR-RFLP pattern of the *lpdA* gene from the 20 strains and accession numbers (Acc. Num.) of *lpdA* gene sequenced

No.	Strain	Serogroup	Serotype	Serosubtype	<i>lpdA</i> RFLP pattern	<i>lpdA</i> sequence Acc. Num.	Strain source ^a	Strain geographical origin	Reference
1	M1080	B	1	P1.1,7	1		MPIMG	Finland	[20]
2	B385	B	4	P1.19,15	1	X77920	IF	Cuba	[6]
3	M992	B	5	P1.1	1		MPIMG	South Africa	[20]
4	M982	B	9	P1.9	1		MPIMG	USA	[20]
5	43-31-1	B	ND	ND	1		CPHE	Cuba	^b
6	H44/76	B	15	P1.7,16	1	X90938	MPIMG	Norway	[20]
7	102/90	B	15	P1.7,16	1		NIPH	Norway	[21]
8	131/91	B	15	P1.7,16	1		NIPH	Norway	[21]
9	140/90	B	15	P1.12	1		NIPH	Norway	[21]
10	51/90	B	15	P1.12	1		NIPH	Norway	[21]
11	41/91	B	16	ND	1		NIPH	Norway	[21]
12	Z222	I	ND	ND	1		MPIMG	China	^c
13	MC51	C	ND	P1.15	1		MPIMG	Norway	[20]
14	M978	B	8	P1.7	2		MPIMG	Dominican Republic	[20]
15	IHN36117	B	14	P1.7	2	X81450	NPHI	USA	[20]
16	2996	B	2b	P1.2	3	X84696	MPIMG	South Africa	[20]
17	M990	B	6	P1.6	3		MPIMG	USA	[20]
18	S3032	B	12	P1.12,16	4	X89748	MPIMG	USA	[20]
19	M136	B	11	ND	5	X89747	MPIMG	USA	[20]
20	B16B6	B	2a	P1.2	5		MPIMG	Norway	[20]

ND, not determined.

^aThe strains were obtained from: MPIMG, Max Planck Institute for Molecular Genetics, Berlin, Germany; IF, Finlay Institute, Havana, Cuba; CPHE, Provincial Centre for Hygiene and Epidemiology, Havana, Cuba; NIPH, National Institute of Public Health, Oslo, Norway; NPHI, National Public Health Institute, Helsinki, Finland.

^bThis strain was clinically isolated in Cuba in 1980. It was serologically classified at the CPHE (Havana, Cuba).

^cNo publication available (Dr. M. Achtman, personal communication).

strain B385. In all strains a DNA fragment of approximately 4.1 kb was detected. In the case of strain B385, this result was expected since the *lpdA* gene, isolated from the EMBL 3 phage library, was contained within a *Eco*RI/*Hind*III chromosomal fragment of 4.1 kb [15]. As we achieved the same result in all the strains, we can conclude that the location of these restriction sites around the *lpdA* gene in the genome of the 20 strains is the same. The higher DNA bands detected in all the strains, and specially in the strain B16B6, are the result of incomplete digestion. From this experiment, we conclude that the *lpdA* gene is present in the 20 analysed *N. meningitidis* strains. The presence of this gene in many other meningococcal strains has been previously reported by immunoidentification in a whole cell ELISA [15,19]. Therefore, it seems that *lpdA* is a highly conserved gene, as it is present in the majority of *N. meningitidis* strains examined by us to date.

To verify the expression of the *lpdA* gene, OMV from the 20 strains were tested by Western blot using the mAb 114, which is specific for the P64k protein.

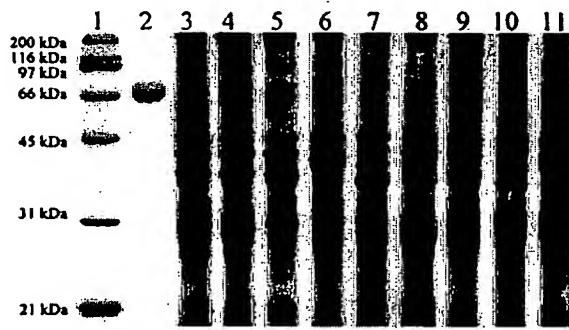


Fig. 2. Molecular mass and antigenicity analysis of P64k protein, using the mAb 114, from eight of the 20 strains analysed in the present study. Lane 1: molecular mass markers, the mass is indicated on the left in kDa. Lane 2: 1 μ g of pure recombinant P64k protein. Lanes 3–11: OMV from the strains (3) *lpdA* mutant strain (negative control), (4) B385, (5) M1080, (6) IHN 36117, (7) M978, (8) M990, (9) 2996, (10) S3032, (11) B16B6. In the upper part the SDS-PAGE analysis is shown, and the Western blotting analysis appears in the lower part.

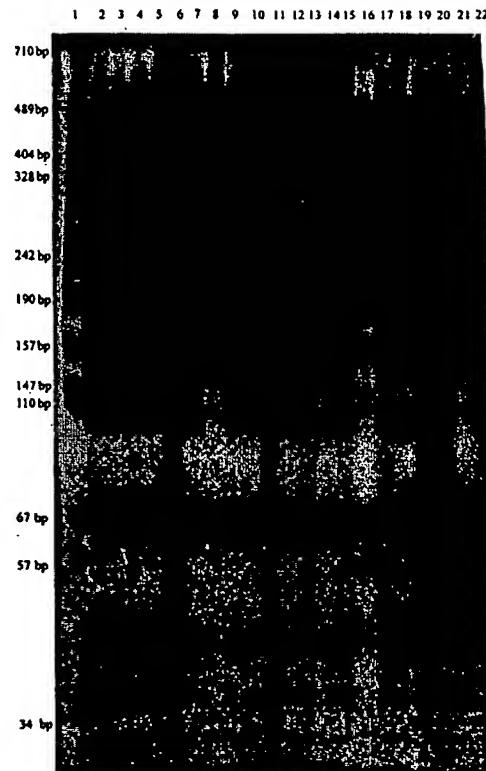


Fig. 3. *Hpa*II RFLP patterns of the *lpdA* gene from the 20 meningococcal strains under study. Lane 2: B385. Lane 3: 43-31-1. Lane 4: M992. Lane 5: M982. Lane 6: IHN36117. Lane 7: Z222. Lane 8: 2996. Lane 9: M1080. Lane 10: MC-51. Lane 11: M978. Lane 12: H44/76. Lane 13: S3032. Lane 14: B16B6. Lane 15: 41/91. Lane 17: 51/90. Lane 18: M136. Lane 19: 102/90. Lane 20: 140/90. Lane 21: M990. Lane 22: 131/91. Lanes 1 and 16: Molecular size markers (pBluescript KS II restricted with *Hpa*II). The sizes are indicated on the left in base pairs.

A high molecular mass protein, equivalent to the P64k of strain B385, was recognised in all the strains. Fig. 2 shows the results obtained. The antigenic conservation is a remarkable characteristic of the P64k protein. The mAb 114 was able to recognise the protein in the 20 different strains. This is an advantage of the P64k protein in order to be considered as a vaccine candidate over other meningococcal proteins, which are antigenically variable [11–13]. For a further characterisation of P64k antigen in terms of gene identity, the strains were differentiated on the basis of RFLP patterns obtained after PCR amplification of the *lpdA* gene and DNA restriction endonuclease digestion.

A

B385	1	MALVELKVPDIGHENVDIIAVEVNVDIATVDDTLITLETDKATMDVPAEVAGVVKEVK
H44/76	1	MALVELKVPDIGHENVDIIAVEVNVDIATVDDTLITLETDKATMDVPAEVAGVVKEVK
IHN36117	1	MALVELKVPDIGHENVDIIAVEVNVDIATVDDTLITLETDKATMDVPAEVAGVVKEVK
2996	1	MALVELKVPDIGHENVDIIAVEVNVDIATVDDTLITLETDKATMDVPAEVAGVVKEVK
S3032	1	MALVELKVPDIGHENVDIIAVEVNVDIATVDDTLITLETDKATMDVPAEVAGVVKEVK
M136	1	MALVELKVPDIGHENVDIIAVEVNVDIATVDDTLITLETDKATMDVPAEVAGVVKEVK

B385	61	VKGVDKISEGGLIVVVEAEGTAAAPKAEAAAAPQEAAPKAAAAPQAAQFGGSADAEDV
H44/76	61	VKGVDKISEGGLIVVVEAEGTAAAPKAEAAAAPQEAAPKAAAAPQAAQFGGSADAEDV
IHN36117	61	VKGVDKISEGGLIVVVEAEGTAAAPKAEAPKAAAAPQEAAPKAAAAPQAAQFGGSADAEDV
2996	61	VKGVDKISEGGLIVVVEAEGTAAAPKAEAPVAPAQEAAPKAAAAPQAAQFGGSADAEDV
S3032	61	VKGVDKISEGGLIVVVEAEGTAAAPKAEAAAASQEAAPKAAAAPQAAQFGGSADAEDV
M136	61	VKGVDKISEGGLIVVVEAER-AASPKAAPVAPAQEAAPKAAAAPQAAQFGGSADAEDV

B385	121	VVLGGPGGYSAAFAAADEGLKVAIVERYKTLGGVCLNVGCP SKALLHNAAVIDEVRHL
H44/76	121	VVLGGPGGYSAAFAAADEGLKVAIVERYKTLGGVCLNVGCP SKALLHNAAVIDEVRHL
IHN36117	121	VLCGG-PGGYSAAFAAADEGLKVAIVERYKTLGGVCLNVGCP SKALLHNAAVIDEVRHL
2996	121	VVLGGPGGYSAAFAAADEGLKVAIVERYKTLGGVCLNVGCP SKALLHNAAVIDEVRHL
S3032	121	VVLGGPGGYSAAFAAADEGLKVAIVERYKTLGGVCLNVGCP SKALLHNAAVIDEVRHL
M136	120	VVLGGPGGYSAAFAAADEGLKVAIVERYKTLGGVCLNVGCP SKALLHNAAVIDEVRHL

B385	181	AANGIKYPEPELDIDMLRAYKDGVVSRLLTGGLAGMAKS RKV DVIQGDGQFLDPH HLEVSL
H44/76	181	AANGIKYPEPELDIDMLRAYKDGVVSRLLTGGLAGMAKS RKV DVIQGDGQFLDPH HLEVSL
IHN36117	180	AANGIKYPEPELDIDMLRAYKNGVVSRLLTGGLAGMAK RKR KV DVIQGDGQFLDPH HLEVSL
2996	181	AANGIKYPEPELDIDMLRAYKNGVVSRLLTGGLAGMAK GRK KV DVIQGDGQFLDPH HLEVSL
S3032	181	AANGIKYPEPELDIDMLRAYKNGVVSRLLTGGLAGMAK GRK KV DVIQGDGQFLDPH HLEVSL
M136	180	AANGIKYPEPELDIDMLRAYKNGVVSRLLTGGLAGMAK GRK KV DVIQGDGQFLDSH HLEVSL

B385	241	TAGDAYEQAAPTGEKKIVAFKNCIIAAGSRVTKLPFIP EDPRIIDSSGALALKEVPGKLL
H44/76	241	TAGDAYEQAAPTGEKKIVAFKNCIIAAGSRVTKLPFIP EDPRIIDSSGALALKEVPGKLL
IHN36117	240	TAGDAYEQAAPTGEKKIVAFKNCIIAAGSRVTKLPFIP EDPRIIDSSGALALKEVPGKLL
2996	241	TAGDAYEQAAPTGEKKIVAVKNCIIAAGSRVTKLPFIP EDPRIIDSSGALALKEVPGKLL
S3032	241	TAGDAYEQAAPTGEKKIVAFKNCIIAAGSCVTKLPFIP EDPRIIDSSGALALKEVPGKLL
M136	240	TAGDAYELAAPTGEKKIVAFKNCIIAAGSRVTKLPFIP EDPRIIDSSGALALKEVPGKLL

B385	301	IIGGGIIGLEMGTVYSTLGSRLDVVEMMDGLMQGAD RDLV KV WQKQNEYRFDNIMVNTKT
H44/76	301	IIGGGIIGLEMGTVYSTLGSRLDVVEMMDGLMQGAD RDLV KV WQKQNEYRFDNIMVNTKT
IHN36117	300	IIGAGIIGLEMGTVYSTLGSRLDVVEMMDGLMQGAD RDLV KV WQKQNEYRFDNIMVNTKT
2996	301	IIGGGIIGLEMGTVYSTLGSRLDVVEMMDGLMQGAD RDLV KV WQKQNEYRFDNIMVNTKT
S3032	301	IIGGGIIGLEMGTVYSTLGSRLDVVEMMDGLMQGAD RDLV KV WQKQNEYRFDNIMVNTKT
M136	300	IIGGGIIGLEMGTVYSTLGSRLDVVEMMDGLMQGAD RDLV KV WQKQNEYRFDNIMVNTKT

B385	361	VAVEPKEDGVYVTFEGANAPKEPQRYDAVLVAAGRAPNGKLISAEKAGVAVTDRGFIEVD
H44/76	361	VAVEPKEDGVYVTFEGANAPKEPQRYDAVLVAAGRAPNGKLISAEKAGVAVTDRGFIEVD
IHN36117	360	VAVEPKEDGVYVTFEGANAPKEPQRYDAVLVAAGRAPNGKLISAEKAGVAVTDRGFIEVD
2996	361	VAVEPKEDGVYVTFEGANAPKEPQRYDAVLVAAGRAPNGKLISAEKAGVAVTDRGFIEVD
S3032	361	VAVEPKEDGVYVTFEGANAPKEPQRYDAVLVAAGRAPNGKLISAEKAGVAVTDRGFIEVD
M136	360	VAVEPKEDGVYVTFEGANAPKEPQRYDAVLVAAGRAPNGKLISAEKAGVAVTDRGFIEVD

Fig. 4. Deduced amino acid sequence alignment of P64k protein from strains: B385, H44/76, IHN36117, 2996, S3032 and M136. Asterisks indicate identical amino acids. Dots indicate homologous amino acids. Numbers on the left indicate the positions of amino acids in the mature protein. Boxed appears the epitope recognised by the mAb 114. The nucleotide sequence of the *lpdA* gene of these strains appears in the EMBL Nucleotide Sequence Database under the accession numbers indicated in Table 1.

B

B385	421	KQMRTNVPHIYAIQDIVGQPMLAHKAVHEGHVAAENCAGHKAYFDARVIPGVAYTSPEVA
H44/76	421	KQMRTNVPHIYAIQDIVGQPMLAHKAVHEGHVAAENCAGHKAYFDARVIPGVAYTSPEVA
IHN36117	420	KQMRTNVPHIYAIQDIVGQPMLAHKAVHEGHVAAENCAGHKAYFDARVIPGVAYTSPEVA
2996	421	KQMRTNVPHIYAIQDIVGQPMLAHKAVHEGHVAAENCAGHKAYFDARVIPGVAYTSPEVA
S3032	421	KQMRTNVPHIYAIQDIVGQPMLAHKAVHEGHVAAENCAGHKAYFDARVIPGVAYTSPEVA
M136	420	KQMRTNVPHIYAIQDIVGQPMLAHKAVHEGHVAAENCAGHKAYFDARVIPGVAYTSPEVA

B385	481	WVGTELASKASGRKITKANFPWAAASGRAIANGCDKPFKLI
H44/76	481	FKLI
IHN36117	480	FKLI
2996	481	FKLI
S3032	481	FKLI
M136	480	FKLI

B385	541	GDMIGEVCLAIEMGCDAA
H44/76	541	DIGKTIH
IHN36117	540	HPTLGE
2996	541	SIGMAAEVALGT
S3032	541	CDLPPQKK-
M136	540	GDMIGEVCLAIEMGCDAA
		DIGKTIH
		HPTLGE
		SIGMAAEVALGT
		CDLPPQKK-
		GDMIGEVCLAIEMGCDAA
		DIGKTIH
		HPTLGE
		SIGMAAEVALGT
		CDLPPQKK-

Fig. 4 (continued).

The PCR-RFLP analysis of the *porA* gene (encoding class 1 protein) has been used by Kertesz et al. [36] for characterising meningococci. The authors of this work claim that their method offers an alternative to characterise isolates that could not be subtyped with monoclonal antibodies. Similarly Peixuan et al. [37] found this method very useful in the epidemiological investigation of group A meningococcal meningitis in China.

In our case, we wanted to use this method to study the diversity of a specific gene, because it has several advantages over other techniques. For example, random errors in nucleotide incorporation by the *T. aquaticus* DNA polymerase during the PCR do not affect RFLP analysis of amplified DNA because the molar prevalence of any single error is too low [38].

For the amplification of the *lpdA* gene from the 20 strains, the same conditions and the same oligonucleotides were used. A PCR product of approximately 1.8 kb, which is the length of the coding region of the *lpdA* gene in the strain B385, was obtained in all the strains (data not shown). This result was expected based on those previously obtained in the Southern and Western blot experiments. For the RFLP study, we chose the enzyme *Hpa*II because it was the enzyme which cut the *lpdA* gene of strain B385 more times among the 325 restriction enzymes

examined, using computer restriction analysis software [39]. Fig. 3 shows the *Hpa*II RFLP patterns obtained. All the strains gave a restriction pattern of approximately 11 *Hpa*II fragments and showed the same pattern of minor bands. Only five different RFLP patterns (named 1–5) were found among the 20 strains. Table 1 summarises the results obtained using this method, which was applied to 18 serogroup B, one serogroup C, and one serogroup I strains (see Table 1). The RFLP pattern 1 was predominant and was found in the *lpdA* gene from 13 of the 20 strains belonging to three different serogroups, seven different serotypes and six different serosubtypes. The RFLP patterns 2, 3 and 5 were equally distributed among the rest of the strains analysed. The strain S3032 showed a unique *Hpa*II RFLP pattern (pattern 4). The five *Hpa*II RFLP patterns obtained in the strains studied were relatively uniform, suggesting the high degree of conservation of this gene at the molecular level.

To verify this hypothesis, the *lpdA* gene of one representative strain of each of the five RFLP patterns obtained was fully sequenced, and the nucleotide and deduced amino acid sequences were compared with the *lpdA* gene and P64k protein sequences of the strain B385. The strains chosen were H44/76, IHN36117, 2996, S3032, and M136; thus, we com-

pared six sequences in total. The accession numbers of the *lpdA* genes sequenced are shown in Table 1. The nucleotide sequences showed 93–99% identity and the deduced aa sequences were 97–99% identical; therefore, we can conclude that this antigen has a very high degree of sequence conservation. The results of the deduced aa sequence comparison are shown in Fig. 4.

It is interesting to note that the minor changes detected at the aa level did not affect the nature of the protein in terms of molecular mass, as could be observed in the Western blotting assay presented in Fig. 2. The peptide recognised by mAb 114 [35] is 100% identical in the P64k protein of the six sequenced strains (boxed in Fig. 4). This may explain why this mAb recognised the protein in these strains when assayed in Western blotting (Fig. 2). We consider that the epitope recognised by this mAb should also be well conserved in the remaining non-sequenced strains.

From this study we can conclude that P64k protein is a stable antigen expressed by many strains of *N. meningitidis*. By genetic analysis we found that the *lpdA* gene is highly conserved among *N. meningitidis* strains. This finding provides us with more arguments for considering the P64k protein as a potential vaccine candidate against meningococci.

In the present work, we have also shown that the PCR-RFLP method, which exploits genetic rather than phenotypic variability, provides a convenient and rapid alternative to the study of gene identity in *Neisseria* species.

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Exhibit D

13/5/79

Transformación de E. Coli (cepa W3110)
con PMTBF y PMEGF cepa MC1061 18/5/79

- ① Descongela una aliquota de células competentes en hielo
- ② Se toman 125 μ l de célula y se pone a dos viales
 - * Siempre se incluye 1 μ l de célula solamente sin DNA.
- ③ Se añaden ≤ 1 μ g de DNA
 - * No saber la tasa 1:1 la que establece plasmido y toma 1 μ l de cada PMEGF y PMTBF
- ④ Se deja en hielo de 10' a 30'
- ⑤ Se le da un choque térmico 2' a 42°C
- ⑥ Se deja 10' en hielo
- ⑦ Se añade 1 ml de LB y se incuban 1 hora a 37°C
- ⑧ Plaqueo, en LBA (omnipedium) 100 μ l de suero
 - Se deja O/N

(Las plaques a 4°C se pueden utilizar hasta 10 días).

17/5/79

se toma una colonia y la sembra en un tubo con 5 ml de LB + 5 ml de Karami (circular).

A las 6:00 horas.

Realmente se toma a las 7:00 horas.

5:00 pm: se saca el cultivo en Zeranda en LB.

O.N.

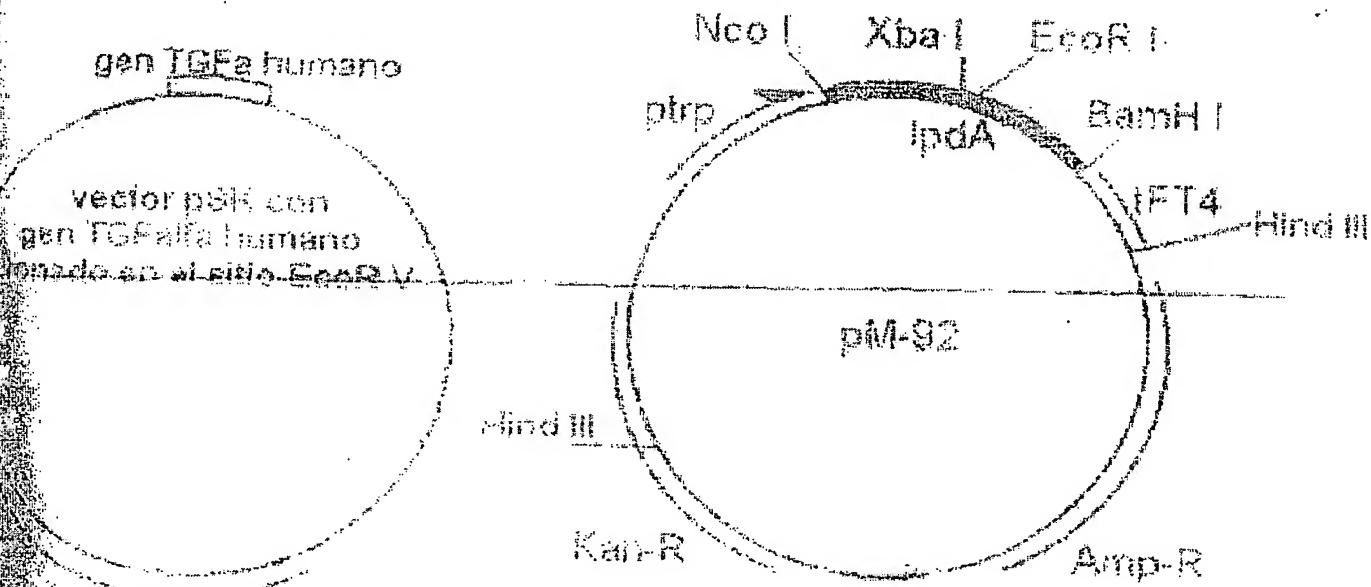
18/5/79

31000 form.

Casi el precipitado

ESQUEMA DE LA CONSTRUCCIÓN DE
DE FUSIÓN PGK-TGFβ HUMANO

207E12



PCR usando una
cadenita de bases
de 18 nucleótidos

Double Injector
Kba I / Eco R I

Extracto de células
Hela o 293T

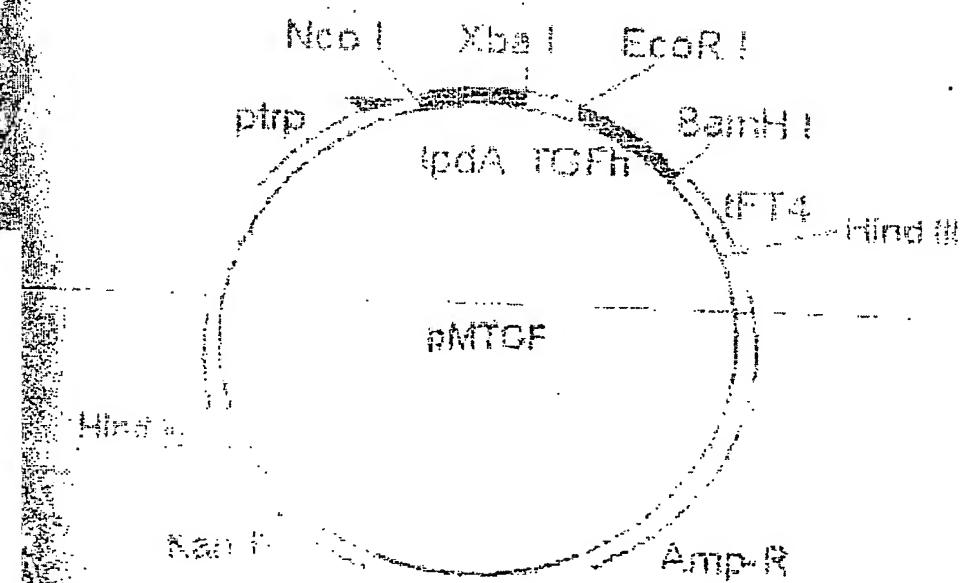


Exhibit E

13/08/99

Controles de las placas. Cinética de la expresión del PNTGF en E.C.1061

SDS PAGE (100% acetonato de

1:30 a 99 Volt (600 mcaz. bandas)

120 Volt (600 mcaz. bandas)

① - PMTGF-MC1061 6/8/99 (100 ul paseo de 60-200 ml LBK
0.1% O.N., clorina y 350 ml)

② - t: 0 LBK ③ t: 6 horas LBK ④ t: 8 horas LBK

⑤ - t: 10 LBK ⑥ t: 12 horas LBK ⑦ t: 0 LBA

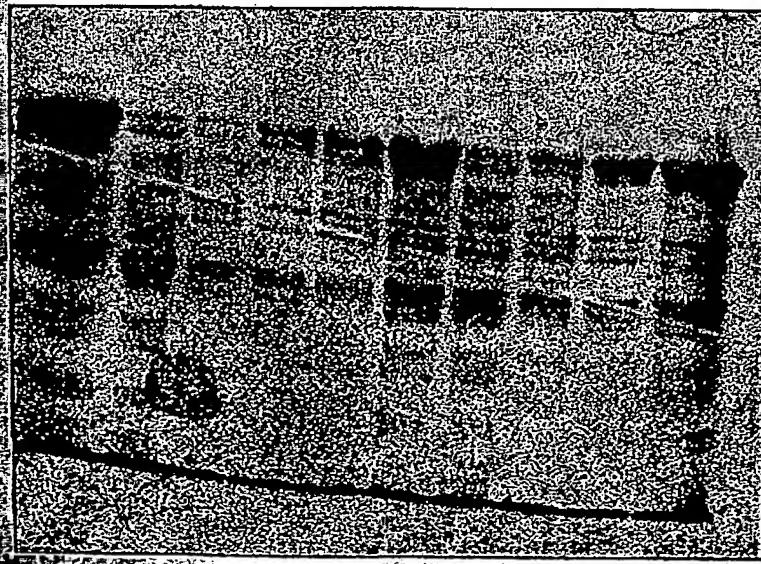
⑧ - t: 8 LBA ⑨ t: 10 LBA ⑩ t: 12 LBA

los otros campos no los incluí porque no me daba la cantidad de muestra ya que la D.O. es muy baja y para aplicar el mismo nº de círculos hace falta tener mas inf.

me fui a: ② → ⑨ → 50 ul + 25 ul
H2O + BS reducido 4X.

aplicar 10 ul de ab.

(K)	0	6	8	10	12	(L)	0	6	10	12
Ctrl 1	2	3	4	5	6	7	8	9	10	



Cinética de la expresión de la proteína fusionada TGFalpha-P64K expresada en la cepa de E.Coli MC1061. A las muestras se les determinó la D.O. a 600 nm y se aplicó 10 ul de cada muestra preparada ([ml de muestra = 2/D.O. (pelle)]) + 50 ul H2O + 25 ul BS reducido 4X) después de 30 min a 100 °C.

1: PMTGF(6/8/99)O.N. 2: tiempo 0 LBK, 3: 6 horas LBK, 4: 8 horas LBK, 5: 10 horas LBK, 6: 12 horas LBK, 7: 0 horas LBA, 8: 8 horas LBA, 9: 10 horas LBA, 10: 12 horas LBA.